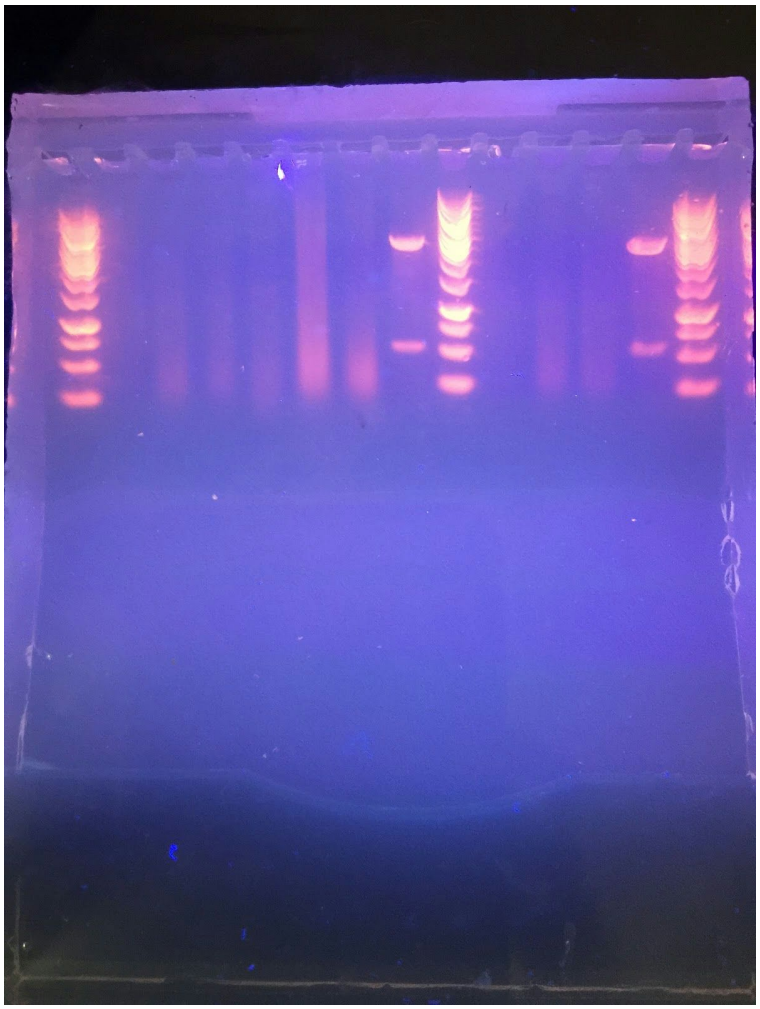
Laura's Physical Notebook A (Starting on page 39)

5/26/2015 LAURA

- CBDA Synthase with Biobrick additions ordered from IDT by Dr. Brewer
- Forward and Reverse Primers also ordered from IDT by Dr Brewer

- Today, I am attempting to verify some samples of possibly ligated PGAPC + L + Mambalgin left by a member of GSUiGEM 2014 team. PCR of samples and restriction digests (XHOI and BAMHI) of 6 samples and also of a previously known PGAPC + L as control We want to see if we can use these
- Restriction digests did not come out as expected for any of the samples of PGAPC + L + Mamba
- 
- Lane 6 and 7 look like there might be something there but streaked bad
- Lane 8 and 13 were control sample of PGAPC+L (no mamba)
- I will be running the PCR samples on a 1% agarose gel 5/27/2015

6/8/2015 LAURA

- Attempting to ligate linearized PSB1C3 with Mamba CDNA
- attempting to ligate linearized PSB1A3 with Mamba CDNA
- Both procedures were done as described on the igem protocols list, minus the DpnI as we do not have that in stock. This enzyme would decrease appearance of red colonies. Decided to go forward and eliminate this step to see if I can still get colonies of my transformation

Using the Linearized Plasmid Backbones

The DNA Distribution should come with a set of linearized plasmid backbones: pSB1A3, pSB1C3, pSB1K3.m1, and pSB1T3. The linearized plasmid backbones (25ng/ul at 50ul) should be stored at 4C or lower. Prior to ligation the plasmid backbones need to be cut with EcoRI and PstI.

Digest

- Enzyme Master Mix for Plasmid Backbone (25ul total, for 6 rxns)
    - 5 ul NEB Buffer 2
    - 0.5 ul BSA
    - 0.5 ul EcoRI-HF
    - 0.5 ul PstI
    - 0.5 ul DpnI (Used to digest any template DNA from production)
    - 18 ul dH20
- Digest Plasmid Backbone
    - Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)
    - Add 4 ul of Enzyme Master Mix
    - Digest 37C/30 min, heat kill 80C/20 min

Ligation

- Add 2ul of digested plasmid backbone (25 ng)
- Add equimolar amount of EcoRI-HF SpeI digested fragment (< 3 ul)
- Add equimolar amount of XbaI PstI digested fragment (< 3 ul)
- Add 1 ul T4 DNA ligase buffer. **Note:** Do not use quick ligase
- Add 0.5 ul T4 DNA ligase
- Add water to 10 ul
- Ligate 16C/30 min, heat kill 80C/20 min
- Transform with 1-2 ul of product

Note: For linearized plasmid backbones provided by iGEM HQ, a plasmid backbone with an insert of BBa_J04450 was used as template. As a result any red colonies that appear during your ligation may be due to the template as a background. Digesting with Dpn1 before use should reduce this occurrence.

- I plated the resulting transformations on appropriate plates (i.e. Chlor plates with psb1c3 cells and amp plates with psb1a3 cells) 150 uL each plate

- will see if I have colonies tomorrow 6/9/15

6/9/2015 LAURA

No growth on plates will re-evaluate and try other protocols next week.

6/16/2015 LAURA

- Decided to Gel isolate the linearized psb1c3, psb1a3, and mambalgin DNA post digest.
- Samples labeled in “Reza’s box” as follows
- 2015616LLI GE1 (Psb1c3 cut with EcoRI and PstI then gel isolated)
- 2015616LLI GE2 (psb1a3 cut with EcoRI and PstI then gel isolated)
- 2015616LLI GE3 (Mamba cDNA cut with EcoRI and PstI then gel isolated)
- Stored in -20 C for future ligation attempts

CBDA Synthase cDNA was delivered to the lab today! Promptly resuspended as per the directions enclosed. Resulted yield was 20ul of 10ng/ul.

I then PCR’d 4 samples of 1 ul each following protocol.

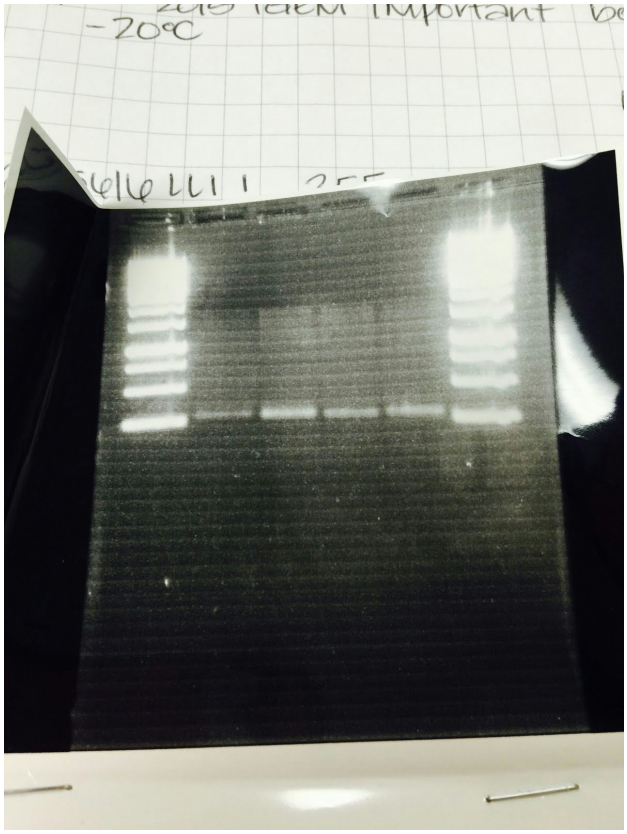
- 12.5 ul Master Mix
- 14.5 ul dH20
- 1 ul CBDA Synthase cDNA (10 ng/ul)
- 1 ul Forward Primer
- 1 ul Reverse Primer

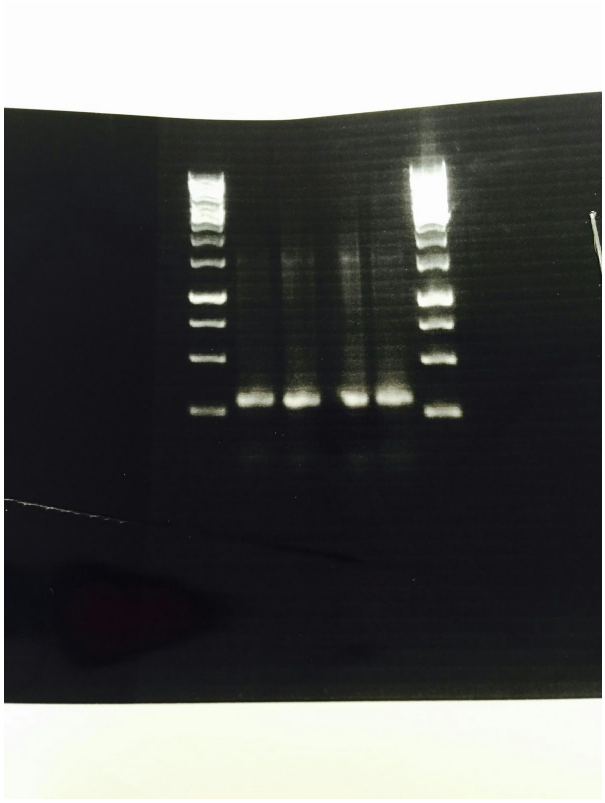
6/17/2015 LAURA

I worked with Grace P today. Checking concentrations of the CDBA Synthase PCR products as follows

- 2015616LLI 1 255 ng/ul
- 2015616LLI 2 450 ng/ul
- 2015616LLI 3 345 ng/ul
- 2015616LLI 4 358 ng/ul

We proceeded to cut a portion of these samples with BcuI and XbaI and ran on a 1% agarose gel.
The results of the gel were less than thrilling.





We will run only PCR product on gel tomorrow to confirm this was a bad result.

6/18/2015 LAURA

Attempted ligations of Mamba cDNA and ECFP and EYFP into linearized psb1c3 (one sample of Mamba into psb1a3) were done today.

Following the protocol as follows.

2 uL T4 Buffer
1mL T4 ligase
2 uL Vector (psb1c3 or psb1a3)
6 uL Insert
9uL dH2O

= 20 uL reaction

Ligated at 22C for 15 minutes
Heat terminate reaction at 65C for 10 minutes

Transformation

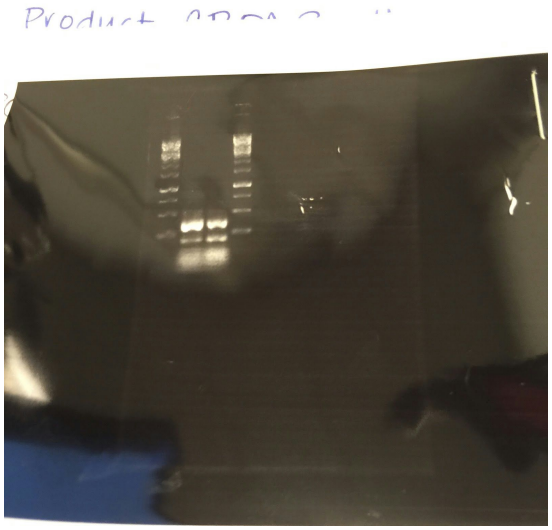
We then took 1 uL of the ligation product and electroporated 50 uL of Big Easy Competent cells Cells were let to recover in 1 mL of sterile SOC for 1 hour at 37C in shaking incubator.

Promptly plated 150 uL per plate with appropriate resistance and let incubate over night at 37C.

PCR Product of CBDA Synthase

On 1 percent agarose gel, I ran the PCR product of CBDA Synthase with loading dye only.

Results as pictured.



The primers used were VF2 and VR. It seems to not be completely amplifying the entire cDNA. Other members of the team are working on creating new constructs with extra base pairs added after the iGEM standard BioBrick prefix and suffix, along with new primers to alleviate this dilemma. For now, I will hold off on more work with this until we have the new constructs in stock for use.

6/22/2015 LAURA

BAMHI

Sample:	Library: Spring 2015 Source Plates, Plate: SP 8009, Well: 6G
Target part:	BBa_I716210 (639 bp) Linked from part info page

COMT

Sample:	Library: Spring 2015 Source Plates, Plate: SP 8010, Well: 2A
Target part:	BBa_I742107 (1101 bp) Linked from part info page

Heme oxygenase

Sample:	Library: Spring 2015 Source Plates, Plate: SP 8011, Well: 9H
Target part:	BBa_I15008 (751 bp) Linked from part info page

Firefly luciferase

Sample:	Library: Spring 2014 Source Plates, Plate: SP 7014, Well: 10B
Target part:	BBa_I712019 (1653 bp) Linked from part info page

These iGEM registry parts were transformed into 40 uL of Big Easy Cells by electroporation

Let to recover in SOC for 1 hour at 37C shaking incubation. Plated on Chloremphenocol resistance plates and let rest overnight in the 37C incubator.

Restriction Digest and GEL extract of psb1k3 linearized backbone

In hopes to test the proposed bacterial promoter for the constructs, we will take some registry parts and test them out. I am planning to attempt a 3A Assembly with the bacterial promoter that Tran has miniprepped and one of the previous kit parts that we have transformed. In doing so, we will need prepared linearized backbone.

I took 8 uL of Linearized Psb1k3
5uL CutSmart Buffer
1 uL PST - HF
1 uL ECORI - HF
35 uL dH2O
= 50 uL reaction

this was left to incubate at 37C for 1 hour.

Next, I ran it on the gel and extracted according to ThermoScientific protocols and reagents.

Eluted with 30 uL Elution Buffer and stored in the -20C.

Labeled as 2015622LI K1 and 2015622LI K2

6/23/2015 LAURA

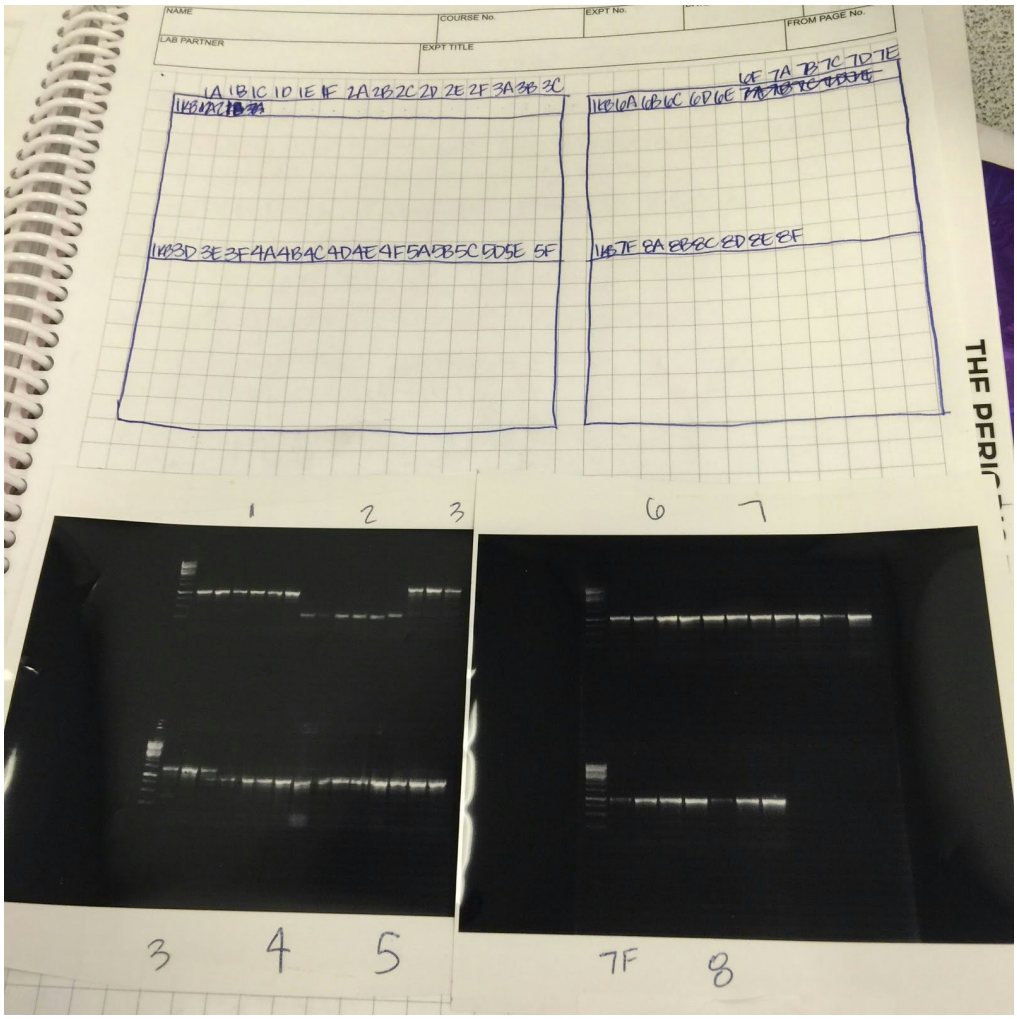
After transformations from yesterday, Grace and I will be working together to colony PCR and overnight culture the growth on the plates.

1/ Heme Oxygenase BBa_I15008
2/ ECFP
3/ COMT BBa_I742107
4/ BamHI Enzyme BBa_I716210
5/ GPA4 BBa_K1184000
6/ EYFP
7/ GPA3 BBa_K1033916
8/ GPA2 BBa_K592100

We picked 6 colonies from each plate to PCR and Overnight culture

PCR reaction setup for 48 reactions (50X)

Colony cells (Per pcr tube)
50 uL Forward Primer (VF2)
50 uL Reverse Primer (VR)
775 uL dH2O
625 uL Master Mix



PCR results as above. It turns out that sample 2 was not what was expected so these results were tossed out as we did not successfully ligate. Every other colony was confirmed by expected base pairs.

6/24/2015 LAURA

Filled out About our lab form for iGEM.org wiki page. Submitted and reviewed with Maruf.

Overnight cultures were all cloudy. :)
Made glycerol stocks of the following samples and were labeled as follows and stored in -80C.

1/ 2015624LI 1F
PSB1C3 + HO1
2/ 2015624LI 2E
PSB1C3 + ECFP
3/ 2015624LI 3B
PSB1C3 +COMT
4/ 2015624LI 4A
PSB1C3 + BamHI
5/ 2015624LI 5A
PSB1C3 + Killer Red
6/ 2015624LI 6D
PSB1C3 + mtag
7/ 2015624LI 7D
PSB1C3 + Lime
8/ 2015624LI 8E
PSB1C3 + EYFP

Mini preps attempted of the previous samples

1st attempt did not return any levels of concentration

I took new samples and grew them up for a few hours in a day culture and attempted again, this time in 10 mL cultures.

1A
3A
4C
5B
6F
7B
8B

The subsequent mini prep also showed poor poor sample concentrations. The miniprep kits are in question.

I will grow another overnight culture with the same samples and attempt again in the morning.

6/25/2015 LAURA

Advisor Maruf suggested a few alterations to the miniprep protocol that may help my results.
1/ Wash step 1 (perform twice)
2/ Use of Midi prep reagents in the Thermo Scientific kit
3/ Heated Elution Buffer (50C)

I did the previous adjustments on the samples (from 10 mL of culture)

I still received little to no concentration of DNA in my samples. :(

I wonder if the nano spec is acting up, RNAse buffer, or if my technique is really that bad.
Will run the newest samples on a gel.
10 uL plus loading dye

6/29/2015 LAURA

- We went to Jess's Lab to watch a Yeast transformation
- First we made fresh YPD and suction filtered instead of autoclaving
- We then inoculated 15 mL with the 5 mL overnight yeast culture prepared the night before
- OD 600 was brought to 0.5 from 0.1
- We also made a stock solution of single stranded Salmon sperm DNA. 2 mg/mL will need to be boiled before use.
- Once the OD reached 0.5 (4:16PM measured at 0.534) we pelleted the cells for 5 minutes at 3500 rpms.
- next, we removed the YPD from the pellet carefully and added 4 mL of TE buffer. Resuspended pellet in TE, then put back in centrifuge to pellet again.
- Next, we removed TE buffer from pellet carefully, and added 4 mL of LiOAc-TE Buffer
- Resuspend pellet and then re-pellet. Remove this LiOAc-TE buffer.
- Next we add 100 uL of LiOAC-TE buffer and resuspend.
- In a new eppendorf tube we put in
- 1 uL Linearized plasmid
- 1.5 uL Single stranded DNA (Boiled)
- 25 uL competent Yeast
- 200 uL PEG
- This is all put in a 30C bath for one hour
- then 42C for twenty minutes
- Then plated on appropriate selection plates

I also worked on re-culturing colonies grown from the previous week as follows

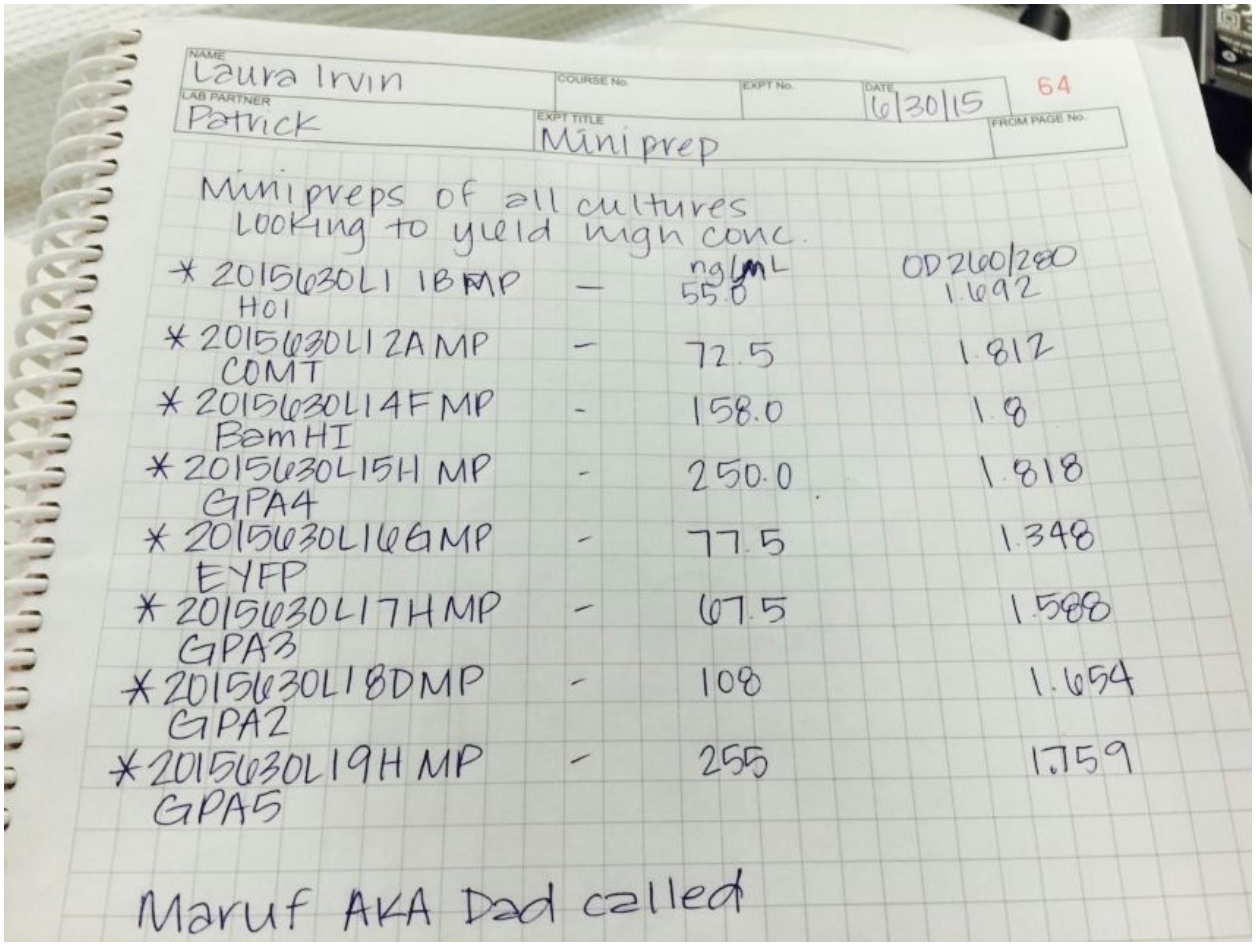
2015629LI 1 B - Heme Oxy

2015629LI 2A - COMT
2015629LI 4F - BamHI
2015629LI 5H - GPA4
2015629LI 6G - EYFP
2015629LI 7H - GPA3
2015629LI 8D - GPA2
2015629LI 9H - GPA5

Cultured overnight in 10 mL of LB + Chlor
also Glycerol Stocks were made with 50% Glycerol at a 1:1 ratio

6/30/2015 LAURA

Miniprepped all cultures with intents of yielding a high concentration


NAME Laura Irvin | COURSE No. | EXPT No. | DATE 6/30/15 | 64
LAB PARTNER Patrick | EXPT TITLE Miniprep | FROM PAGE No.

Minipreps of all cultures
Looking to yield high conc.

		ng/mL	OD 260/280
* 2015630LI 1B MP HOI	–	55.0	1.692
* 2015630LI 2A MP COMT	–	72.5	1.812
* 2015630LI 4F MP BamHI	–	158.0	1.8
* 2015630LI 5H MP GPA4	–	250.0	1.818
* 2015630LI 6G MP EYFP	–	77.5	1.348
* 2015630LI 7H MP GPA3	–	67.5	1.588
* 2015630LI 8D MP GPA2	–	108	1.654
* 2015630LI 9H MP GPA5	–	255	1.759

Maruf AKA Dad called

After these Nanodrops were performed. I started prep for Electro Comp Cells (Culture XL1 B labeled 2015630LI XL1B, Autoclaved appropriate supplies and tips)

7/1/2015 LAURA

-Started 500 mL of competent cell growth, optimal OD 550 to reach just under 0.8.

Also while waiting on cell growth, started 3A Assembly as per iGEM website protocol.

Master Mixes were prepared

I decided to use Tran's Promoter along with two cDNA's that we miniprepped the day before.

C1- COMT (72.5 ng/uL)
C2 - EYFP (77.5 ng/uL)

PSB1K3 was used for the backbone. (Kanamycin resistence)

All parts were cut and combined with T4 ligase as directed and let incubate for the appropriate amount of time.

Ligation 1 --
Electroporated 1 uL of ligation product (Promoter and COMT with PSB1K3) with 40 uL of XL1B cells

Ligation 2 --
Electroporated 1 uL of ligation product (Promoter and EYFP with PSB1K3) with 40 uL of XL1B cells

Let to recover in SOC at 37C Shaking incubator for at least 1 hour.

100 uL of culture was plated on Kanamycin resistance plates.
201571LI LIG1
201571LI LIG2

These were let to grow overnight in the 37C counter incubator.

__Competent Cells__

	TIME	Flask 1	Flask 2
1st	12:38PM	0.026	0.053
2nd	1:19PM	0.116	0.127
3rd	2:00PM	0.240	0.321
4th	2:30PM	0.439	0.529
5th	2:57PM	0.565	0.709 (Pulled)
6th	3:11PM	0.706	
7th	3:15PM	0.722	
8th	3:19PM	0.789	

4:00PM split into 50 mL Falcon Tubes on ice ; 3900 rpm @4C for 30 minutes
4:30PM Decant LB from each tube, resuspended in 10% Glycerol (Cold)
4:54PM 3900 rpm @4C for 30 minutes ; decant LB and resuspend each tube in 10% glycerol (cold)
Combine pellets to one falcon tube

Aliquot into 120uL portions and flash freeze in ethanol and ice mixture

Store in -80C.

07/06/2015 LAURA

Growth present on Ligation product plates
-201571LI Lig 1

-201571LI Lig 2

Will pick 6 colonies on each plate

- Run Colony PCR
- Run Overnight cultures (10 mL)

Labeled as follows
201571LI COMT1
201571LI COMT2
201571LI COMT3
201571LI COMT4
201571LI COMT5
201571LI COMT6

201571LI EYFP1
201571LI EYFP2
201571LI EYFP3
201571LI EYFP4
201571LI EYFP5
201571LI EYFP6

PCR Cocktail (13 rxns)
10.5 uL dH2O
12.5 uL Master Mix
1 uL VF2
1 uL VR

So… Cocktail is
136.5 uL dH2O
162.5 uL Master Mix
13 uL VF2
13 uL VR

Aliquot into 25 uL portions for each colony.

After running gel

NAME Laura Irvin

LAB PARTNER

COURSE No.

EXPT No.

DATE

EXPT TITLE colony PCR

Not looking promising

11

1 C1 C2 C3 C4 C5 C6 1 E1 E2 E3 E4 E5 E6

07/07/2015 LAURA

All stocks grew in the cultures of LB and Chloramphenicol

According to PCR results, not really expecting to see the sequence I am hoping to see

Chose one mini prep sample from each set of samples
COMT5
EYFP5

glycerol stocks made
201571LI GS EYFP5
201571LI GS COMT5
Will do NotI digest with 10 uL of miniprep product

10 uL DNA
2 uL FD Buffer
0.5 uL NotI
7.5 uL dH2O

Again, results did not give what was expected.

Concluding that 3A assembly did not work.

7/8/2015 LAURA

Minipreps for Sequencing possible PGAPC plus Linker from last year's team

1/ PGAPC + R E.coli 5/17/13 - Labeled 201578LI 1MP 57.5ng/ul
5/ PGAPC Mamba 8/23 - Labeled 201578LI 5MP 138 ng/ul
7/ PGAPZ Alpha A + Linker 6/17/15 DB - Labeled 201578LI 7MP 25 ng/ul
11/ 7/23/13 E. Coli + PGAPZ alpha A plus Linker - Labeled 201578LI 11MP 20 ng/ul
12/ 9/25 E. Coli + PGAPZ alpha A plus Linker - Labeled 201578LI 12MP 32.5 ng/ul
14/ PGAPZ Alpha A with Linker 12/15/13 KV - Labeled 201578LI 14MP 85 ng/ul
16/ PGAPC + L RA 4/7/2015 - Labeled 201578LI 16MP 75 ng/ul

We sent 1, 5, 14, 16 for sequencing with Ping

Labeled as
PGAP1
PGAP5
PGAP14
PGAP16

Later learned that the sequencer is down and Ping is on vacation, these results will be delayed

Restriction Digest of HRP, Mamba, and Alpha Secretion

So, to prep some purified PCR product of each construct for ligation to PSB1C3 vector, we will cut Mamba and Alpha secretion constructs with XbaI and PstI

We will cut the HRP construct with EcoRI and PstI. We will also cut linearized backbone (PSB1C3) with EcoRI and PstI.

Labelled as follows
L1 714 M (Mambalgin)
L1 714 A-S (Alpha Secretion)
L1 714 Hrp (Horseradish Peroxidase)
L1 714 B (PSB1C3 backbone)

50 uL reactions according to protocol for 3 hours at 37C.

Will attempt an overnight ligation of Hrp to PSB1C3
L1 714 LHrp
T4 buffer - 2 uL
Vector (50 ng) - 3 uL
Insert (37.5 ng) - 9 uL
dH2O - 5 uL
T4 Ligase - 1 uL

20 uL Reaction

overnight at 16C

07/15/2015 LAURA

Transformation of ligation product

*sigma 10 DH5Alpha competent cells (Chemically competent)
add 10 uL Ligation product to 40 uL cells
incubated on ice for 30 minutes
Heat Shock in water bath at 42C for 30 - 60 seconds
Add product to 1 mL SOC and shake for 1 hour at 37C

*XL1B Electrocompetent cells
add 1 uL of ligation product to 40 uL of cells
Electroporate - add to 1 mL SOC and shake for 1 hour at 37C

Controls performed

Plated cultured transformations as follows

2015715LI LHrp (Chem)
2015715LI LHrp (Electro)
2015715LI Control (Chem)
2015715LI Control (Electro)

on LB + Chloramphenicol plates

7/16/2015 LAURA

All plates look the same. Inconclusive. Control does not show anything different than the actual ligation plates

Will go at it again on Monday
7/20/2015 LAURA

Verifying with PCR, 3 samples of “Mamba in pSB1C3” found in the 2014 Submission Box.

After quick UV lamp screen, all samples look good and assume to hold Mambalgin-1 with Eco-RI and PstI restriction sites.

Perfomed 4 transformations today.

2 of ligation product (Labelled Lhrp, Horseradish peroxidase in psb1c3) into XL1B electro comp cells

1 of ligation product (Labelled Lhrp, Horseradish Peroxidase in psb1c3) into DH5Alpha Chemically competent cells

1 of #3 Mambalgin-1 in psb1C3 into DH5Alpha Chemically Competent Cells

Plated all on LB + Chlor plates with 1 negative control

7/21/2015 LAURA (Book B)

PCR Screen colonies and Cultures

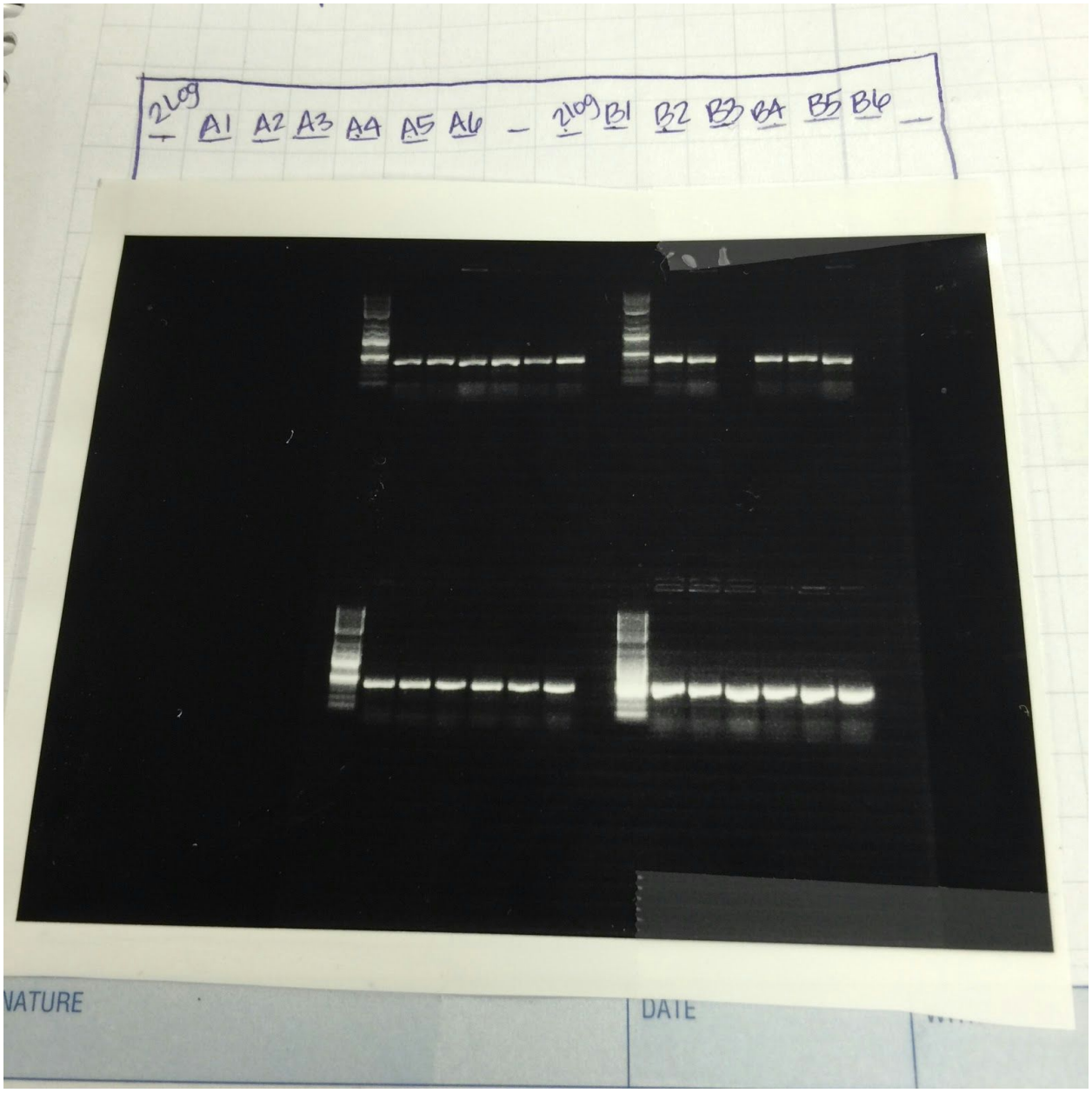
Plates:
A- 2015720LI = LHRP1 Electro trans lig product
B- 2015720LI = LHRP2 Electro trans lig product
C- 2015720LI = LHRP Chem Comp D5H alpha
D- 2015720LI = Psb + Mamba

6 colonies of each labeled 1 - 6

25x PCR Cocktail
250 uL Master Mix
200 uL dH2O
25 uL VR
25 uL VF2

Cells per tube - Appropriately labeled

2 Log Ladder used



07/22/2015 LAURA

Miniprep of samples - Eluted with 40 uL of Elution buffer

2015722LI LHrp A
2015722LI LHrp B
2015722LI LHrp C 25.0 ng/uL
2015722LI Psb1C3 + Mamba 1D
2015722LI Psb1C3 + Mamba 2D

2015722LI Psb1C3 + Mamba 3D

Not sure why I am not getting concentrations of DNA from my Minipreps

07/23/2015 LAURA

Sending for sequencing

MB01 - Hrp in PSB (15) with VR
MB02 - Hrp in PSB (15) with VF2
MB05 - Mamba in PSB with VR
MB06 - Mamba in PSB with VF2
MB07 - Mamba in pGAP with pPic_F
MB08 - Mamba in pGAP with pPic_R

07/28/2015 LAURA

Will grow up more cultures and run PCR on colonies from plate D (Mamba in PSB)
Will try and remember to glycerol stock these cultures for future use.

Samples	Culture volume	PCR Primers Used
2015728LI D7	10 mL	VR, VF2
2015728LI D8	10 mL	VR, VF2
2015728LI D9	10 mL	VR, VF2
2015728LI D10	10 mL	VR, VF2
2015728LI D11	10 mL	M cDNA F, M cDNA R
2015728LI D12	10 mL	M cDNA F, M cDNA R
2015728LI D13	10 mL	M cDNA F, M cDNA R

Verification looked good on PCR - Will miniprep cultures tomorrow

Tobacco Callus

Sterilized biosafety hood with 70% ethanol

Sterilized tools.

Tobacco Callus 1 and 2 were cut up into 5 pieces in the sterile hood with sterile tools and placed gently into the sterile Root and Shoot Media Tubes purchased.

Tubes were then placed in a tray and 16 hour day/8 hour night lighting cycle was set up.

2015728LI Tobacco Callus 1 (A- E)
2015728LI Tobacco Callus 2 (A- E)